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(54) Title: CANCER DIAGNOSIS

(57) Abstract

A diagnostic kit, for determining the ratio of lithocholic acid (LA) to deoxycholic acid (DA) in, say, a faecal sample, comprises a substrate for one acid; a substrate for the other or both acids; an agent which reacts with each substrate to produce a detectable response proportional to the presence of the acid-labelled substrate complex, the relative responses being different in the cases when the LA:DA ratio is normal and substantially higher than normal, respectively.

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CANCER DIAGNOSIS

Field of the Invention

This invention relates to a kit for use in a rapid diagnostic process for determining the ratio of lithocholic acid (LA) and deoxycholic acid (DA) in a faecal extract; these two bile acids have been implicated in human colo-rectal carcinogenesis. This invention has direct application in the preliminary screening of faeces from high risk groups (aged between 35-60) for predicting the likelihood of developing colo-rectal cancer.

Background of the Invention

Large bowel carcinogenesis is a multi-stage process involving the formation and then growth of an adenoma, the development of increasingly severe epithelial dysplasia, and finally the progression to malignancy. There is a substantial amount of evidence incriminating bile acids in colo-rectal carcinogenesis, and the total faecal bile acid concentration is highly correlated with the incidence of large bowel cancer in population studies. However, the correlation is poor in most case-control studies, suggesting that the relationship is more complex. Recent studies suggest that a better discriminant is the ratio of the two principal faecal bile acids, LA and DA; the evidence comes from *in vitro* experiments and from studies of high risk cancer patients and of animals.

Narisawa *et al*, JNCI 53, 1093-1097 (1974), report animal studies showing that the secondary bile acids (LA and DA) were co-carcinogenic in the rat colon, whereas the primary bile acids (cholic and chenodeoxycholic acids) were not.

Owen *et al*, Eur. J. Cancer Clin. Oncol. 19, 1307 (1983), show that a high LA:DA ratio or a very low LA:DA ratio is comutagenic, but more equal mixtures are less

so. Further, the LA:DA ratio increases with adenoma size, i.e. as the malignant potential of the adenoma increases.

Wilpart et al, Gastroenterol. Clin. Biol. 8, 337-342 (1984), used animal experiments to show that increased dietary fat causes a change in the LA:DA ratio, to one which is more comutagenic. Thompson et al, Biochem Soc. Trans. 13 392 (1985), report a similar result in a study of human volunteers on a high-fat diet, and the converse when volunteers were put on a high-fibre diet.

Apart from the effect on dietary fat and fibre, little is known of the factors determining the LA:DA ratio. LA and DA are the products of 7α-dehydroxylation of chenodeoxycholic and cholic acids. Synthesis of chenodeoxycholic acid is reportedly from dietary cholesterol. Thus, the ratio could be a measure of the response to dietary cholesterol; this would support the epidemiological observations of Lui et al, Lancet 2, 784-789 (1979).

Owen et al, supra, conclude that the LA:DA ratio "may be an important aetiological factor in colo-rectal cancer". Further work by Owen et al presents evidence "that the ratio of the two major faecal bile acids, lithocholic and deoxycholic acids, may be a good risk marker"; see Nutrition and Cancer 9 Nos. 2&3, 67-71 (1987). However, the evidence is not conclusive.

It is an object of this invention to provide a rapid and cost efficient methodology to facilitate screening of faecal samples for measurement of the LA:DA ratio as an indication of the potential to contract colo-rectal cancer.

Summary of the Invention

A novel, rapid method for measuring the ratio of lithocholic acid to deoxycholic acid in a sample is based on the use of substrates to LA and DA, respectively. A

novel kit comprises these substrates and an agent which reacts with each substrate to produce a detectable response proportional to the presence of the acid-labelled substrate complex, the relative responses being different in the cases when the LA:DA ratio is normal and substantially higher than normal, respectively.

The value of such a kit has been established by recent, incontrovertible evidence that the LA:DA ratio is a distinctive marker for colo-rectal cancer, including tumours of Duke's grades A, B, C1 and C2, tumours of the caecum and ascending colon, sigmoid and recto-sigmoid colon, and rectum, by comparison with controls, in a study of 78 subjects. Other studies have been confirmatory.

Description of the Invention

The term "substrate" is used herein to describe a material with which LA or DA reacts or binds, i.e. a reacting or binding partner, preferably specifically in context. The material may be, for example, an enzyme for which LA or DA is, in the more usual sense, a substrate.

The substrates may each be specific for one of the two acids. The substrates are, for example, monoclonal or polyclonal antibodies to LA and DA, respectively.

Desired monoclonal antibodies can be prepared in conventional manner. Ultra-pure samples of LA and DA are used as antigens and administered to mice, to obtain antibodies using standard techniques well known to those versed in the art of preparing antibodies. The monoclonal antibodies are recovered, purified and tested for their cross-reactivity with all other bile acids. Monoclonals showing absolute specificity for LA and DA respectively are collected and used in the novel kit.

Desired polyclonal antibodies can also be prepared in conventional manner. The antigens, LA and DA, are injected into sheep, rabbit or donkey as classical routes

to obtaining polyclonal antibodies. The antigens themselves are normally bound in different ways to a larger carrier molecule, one which is relatively inert. Albumin is used frequently. The antigens are bound in such a way so as to expose only specific sites for targets for the antibodies. The bile acids usually only differ in the number and position of hydroxyl groups, e.g. 7α-hydroxyl, 3α-, 12α- etc.

The molecules can be arranged and bound to expose the hydroxyl groups in the 3α and 12α positions or 3α and 7α positions. In this way the immunisation process can lead to the production of very specific polyclonal antibody preparations.

The sheep or other animal is bled after 6-9 months and the best individual animal maintained once the antibodies showing the desired characteristics have been selected. The antibodies are recovered by standard methods of filtration, centrifugation, ammonium sulphate precipitation and chromatographic absorption techniques etc.

The antibodies are purified and tagged with enzymes such as horseradish peroxides or alkaline phosphatase as used in standard ELISA procedures.

The antisera are evaluated for their cross-reactivity against 20-30 related compounds, especially bile acid derivatives. The affinity constants, titre etc. are determined for the individual polyclonal antibodies.

Assay systems are then constructed, setting up competitive and non-competitive methods evaluating reaction time, sensitivity, component stability, kit stability, recovery, precision, matrix, etc.

The antibodies are added to a faecal extract or other suitable sample, and bind specifically to either lithocholate or deoxycholate. After washing, the

LA/DA-bound conjugates are monitored to determine the concentration of the label and thus of LA and of DA. The method is direct and specific for measuring the LA:DA ratio.

The label is, for example, an enzyme or a fluorescent, luminescent, radioactive or ferro-magnetic compound. The most suitable fluorescent probe is fluorescein. In fluorescence polarisation, a labelled sample is excited with polarised light and the degree of polarisation of the emitted light is measured; as antigen binds to the antibody, its rotation slows down and the degree of polarisation increases.

For use in a chemiluminescent, e.g. bioluminescent, assay, light emitted by the bound, labelled antibody is measured. A catalytic protein or enzyme such as luciferase increases the efficiency of the luminescent reaction.

For economy and simplicity, enzyme-linked immunoassays are preferred. The enzyme label is, for example, alkaline phosphatase, glucose oxidase, galactosidase, peroxidase, urease or luciferase. In an enzyme-linked immunosorbent assay, the amount of bound, labelled antibody is determined by recording the light emitted when the enzyme reacts with its substrate to form a chromagen.

In order to determine the respective amount of LA and DA in a given sample, some means for recording a signal associated with the label, e.g. a colorimeter, is used. The sample may be contacted simultaneously with the two polyclonal antibodies, each having a different label generating a distinct signal which can be recorded. Alternatively, a sample may be contacted with the labelled antibodies sequentially, or the sample may be divided and parts contacted with the antibodies separately and simultaneously.

The substrates themselves may be enzymes. For example, they may be respectively substrates for 3α-hydroxy bile acids (including LA and DA) and 12α-hydroxy bile acids (DA but not LA).

More specifically, a kit of the invention may be based on a simple colorimetric method for measuring the specific LA:DA ratio in faeces using an enzymatic technique. The two bile acids are distinguished from the faecal pool of bile acids by using differential assays composed of alpha-hydroxysteroid dehydrogenase (α-HSDH). A sample is assayed for the concentration of 3α-hydroxy bile acids and 12α-hydroxy bile acids using the 3α-HSDH and 12α-HSDH enzyme respectively. The α-HSDH reactions are coupled, via the NADH coproduct, through a second enzyme, diaphorase, which reduces a chromogenic substrate to generate a coloured compound. The increase in absorbance due to this chromagen is directly proportional to the concentration of bile acid present. The difference in absorbance readings between 3α-HSDH and 12α-HSDH reactions is directly proportional to the LA concentration in the faecal sample: the 12α-HSDH reaction quantitates the DA content of the sample.

The specific alpha-hydroxysteroid dehydrogenases (3α-, 7α-, 12α-HSDH's) convert bile acid substrates containing 3α-, 7α-, 12α-hydroxyl groups to the corresponding keto acids and generate reduced cofactor (NADH) concomitantly. The keto products or, preferably, the reduced cofactor product can be measured using chromogenic techniques. For example, the present invention utilises the coupling of a diaphorase-mediated chromogenic system, such as nitrotetrazolium salt, to the products of the α-HSDH reaction.

Means may be provided, for quantitatively assaying the bile acid substrates using buffered reaction mixtures containing α-HSDH enzyme, NAD+, diaphorase and oxidised

chromagen, and following the increase in absorbance spectrophotometrically.

The present invention provides a means of indirectly determining the level of LA by differential assays using α-HSDH's, for which there is no known direct enzyme assay.

A kit of the invention may comprise the respective substrates immobilised on the same or different supports; the sample is then brought into contact with the support(s). Depending on the nature of the label, the support may then be washed and the signal proportional to bound LA or DA recorded following generation of the appropriate signal by contact with a substrate for the label.

A kit of the invention comprises the two substrates, preferably together with an enzyme substrate or other material which can be used to generate a signal associated with the, say, labelled antibody-bile acid reaction product. Means for observing and recording the signal are preferably also provided. The means may discriminate samples from patients which are most at risk, e.g. those in which the LA:DA ratio is unacceptably high, e.g. above 0.8 or 0.9. Risk patients may exhibit a ratio of 0.8 to 1.1, patients already exhibiting cancer a ratio of 1.3 to 4.0.

The sample which is observed may be from faeces, bile juice, urine or blood. Urine may be a marker for colo-rectal bile acid overflow.

There are various ways in which the present invention may be used in practice. For example, the kit may be used for mass screenings. The reactions could be conducted in a microtitre dish (having, say, 500 wells), and the absorbance of washed and reacted labelled antibodies monitored by a large autoscanner. The samples could be treated in duplicate with the same (or

different) chromogenic substrate. The direct ratio of absorbances can be measured. This procedure enables hundreds of samples per day to be screened.

Alternatively, the kit may be used in the form of a simple device for use in a doctor's or nurse's surgery. A patient's faecal sample would have to be extracted. A, say, 1 ml eluate sample can then be split and reacted with the two antibodies separately and their absorbances monitored in a hand-held colorimeter. This could give a result within 30-60 min, allowing immediate doctor/patient discussions.

Further, the novel kit can be used as an OTC aid. An individual could buy a faecal bowel cancer test kit comprising 2 dipsticks coated in labelled antibodies and marked A and B. Each dipstick would be immersed into a fresh faecal sample (on toilet paper) then pushed into a compartmented tube (to wash and react the dipstick). The test would be qualitative, but any major colour difference between the 2 dipsticks would indicate a high ratio effect and the possible need for referral to a doctor.

In either case, the kit of the invention can be used as part of an assay for cancer utilising the substrates etc, and associated with a method of treatment by administering, to a subject having an increased LA:DA ratio, a suitable medicament or dietary regime adapted to reduce the ratio.

The following Example illustrates the invention.

Example

For assay purposes, a kit comprises containers containing given amounts of the reactants used in the following procedure:

A sample of faecal extract is dissolved in a suitable reconstituting buffer (e.g. 50 nM phosphate, Tris, glycine-NaOH) pH 6-9, preferably pH 8. An aliquot

of the sample (e.g. 100 ml) is then mixed with a reaction mixture containing nicotinamide adeninedinucleotide (NAD+), phosphate buffer, nitrotetrazolium blue (or other chromogen), Titron x-100, diaphorase (Clostridium sp. or pig heart enzyme) and 3α-HSDH (from Pseudomonas sp. or another source). The mixture is incubated at 37°C for 15 min. The reaction is stopped using a suitable stopper reagent (e.g. 0.5% sodium dodecylsulphate). During the reaction, the 3α-hydroxyl groups on the bile acids present in the sample will be oxidised to the corresponding keto acids by the 3α-HSDH enzyme. Concomitantly, the NAD+ present is reduced to NADH. The NADH reducing equivalents are then transferred to the chromogenic substrate present by action of the enzyme diaphorase. A typical chromogenic salt (nitrotetrazolium blue) generates a stable blue formazon derivative on the end product. The amount of formazon produced is measured photometrically at 540 nm. The net absorbance obtained (i.e. the difference between the absorbances of the sample and a blank) is directly proportional to the concentration of 3α-hydroxy bile acid present in the sample.

The faecal sample is reacted for a second time, in the same reaction mixture as that described above, except that it contains the enzyme 12α-hydroxysteroid dehydrogenase (12α-HSDH) instead of the 3α-HSDH. After incubation for 15 min at 37°C, the net absorbance obtained from the production of the blue formazon derivative is proportional to the concentration of 12α-hydroxy bile acids present in the sample.

The determination of the LA concentration in the faecal extract is made by subtracting the 12α test result from the 3α test result. The difference is directly correlated with the concentration of LA in the extract as this bile acid contributes to over 98% of the remaining

3α-hydroxy content of faecal bile acid once the 12α-hydroxy bile acids have been assayed.

If desired, the faecal extract can be reacted for a third time, under identical conditions to those described above, except that the mixture contains a specific enzyme, 7α-hydroxysteroid dehydrogenase (7α-HSDH), instead of the 3α- or 12α-HSDH enzyme. After incubation for 15 min at 37°C, the net absorbance obtained from the production of blue formazon (or other chromogenic product) is proportional to the concentration of 7α-hydroxy bile acid present. This provides a check on the LA concentration, giving the maximum percentage error in the LA determination by measuring the cholate and chenodeoxycholate present. It is well known that the concentration of LA and DA in bile fluid is very low or zero whilst, in faecal extracts, the levels of cholate and chenodeoxycholate are very low. Therefore, it is preferred to use only the first two assays.

CLAIMS

1. A diagnostic kit, for determining the ratio of lithocholic acid (LA) to deoxycholic acid (DA) in, say, a faecal sample, comprising a substrate for one acid; a substrate for the other or both acids; an agent which reacts with each substrate to produce a detectable response proportional to the presence of the acid-labelled substrate complex, the relative responses being different in the cases when the LA:DA ratio is normal and substantially higher than normal, respectively.

2. A kit according to claim 1, wherein each substrate is a labelled monoclonal antibody for the respective acid.

3. A kit according to claim 1, wherein each substrate is a labelled polyclonal antibody for the respective acid.

4. A kit according to claim 1, wherein the substrates are respectively for 3α-hydroxy bile acids and 12α-hydroxy bile acids.

5. A kit according to claim 4, wherein the substrates are enzymes.

6. A kit according to claim 5, wherein the enzymes are labelled.

7. A kit according to any of claims 2, 3 and 6, wherein the label is luminescent or chromogenic.

8. A kit according to claim 5, wherein the agent comprises reactants which generate a coloured species when coupled to the respective enzyme-substrate reactions.

9. A kit according to any preceding claim, which comprises means for determining the LA:DA ratio and displaying the different responses.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00294

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 33/574, 33/92, 33/577, C 12 Q 1/00,//C 12 Q 1/32

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N, C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0037742 (NYEGAARD & CO. A/S) 14 October 1981, see abstract; page 2, line 24 - page 3, line 37	1
Y		4,5,8
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Y	Chemical Abstracts, vol. 106, no. 25, 22 June 1987 (Columbus, Ohio, US) see page 349, abstract no. 210560r & JP, A, 61260896 (SEKISUI CHEMICAL CO. LTD) 19 November 1986	4,5,8
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Y	Biochem. Soc. Trans., vol. 12, 1984, 607th Meeting, London (GB) R.W. Owen et al.: "The faecal ratio of lithocholic acid to deoxycholic acid may be an important aetiological factor in colo-rectal cancer", page 861, see page 861	1,3
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Y	Chemical Abstracts, vol. 92, no. 5, 4 February 1980 (Columbus, Ohio, US)	1,3

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
26th June 1989	20. 07. 89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

III. DOCUMENTS CONSIDERED TO BE RELEVANT (C NTINUED FR M THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	O.A. Janne et al.: "Radioimmunoassay of primary and secondary bile acids in serum with specific antisera and iodine 125-labeled ligands", see page 376, abstract no. 37184p & Radioimmunoassay Relat. Proced. Med. Proc. Int. Symp. 1977 (Pub. 1978) 2, 285-93 --	
Y	Nutrition & Cancer, vol. 9, nos. 2,3; 1987 Lawrence Erlbaum Ass. Inc. R.W. Owen et al.: "The importance of the ratio of lithocholic to deoxycholic acid in large bowel carcinogenesis", pages 67-71, see pages 67-70 (cited in the application) --	1,4,5,8
Y	Clinical Chemistry, vol. 26, no. 9, August 1980, Winston-Salem (US) M.J. Crowell et al.: "Enzymic determination of 3α-, 7α-, and 12α-hydroxyl groups of fecal bile salts", pages 1298-1300, see the whole article --	1,4,5,8
A	Gastroenterol. Clin. Biol., vol. 8, 1984, P. Masson, Paris (FR) M. Wilpart et al.: "Lipides alimentaires et variations du débit fécal et des composants métaboliques des selles chez le rat", pages 337-342, see the abstract; page 338, column 2, lines 32-36; page 341, column 2, lines 1-13 (cited in the application)	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8900294 SA 27765

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/07/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0037742	14-10-81	AT-T- E10860	15-01-85
		CA-A- 1163907	20-03-84
		JP-A- 56151499	24-11-81

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82